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GENETICALLY ENGINEERED *ESCHERICHIA COLI* FOR ETHANOL PRODUCTION FROM XYLOSE
Substrate and Product Inhibition and Kinetic Parameters†

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A recombinant strain of *Escherichia coli* FBR5 was characterized for ethanol production from xylose in batch reactors. Up to a salt (NaCl) concentration of 10 gL^{-1}, the culture exhibited no inhibition. Above 10 gL^{-1} of salt concentration the culture experienced inhibition and the maximum concentration of salt that *E. coli* FBR5 could tolerate was 40 gL^{-1}. At 40 gL^{-1} NaCl concentration, the value of μ_{max} (maximum specific growth rate, h^{-1}) was reduced significantly as compared to control where salt concentration was 0 gL^{-1}. The culture could tolerate a maximum xylose concentration of 250 gL^{-1}, however, at that concentration a reduced cell growth was obtained. A maximum cell concentration of 0.30 gL^{-1} was obtained at this sugar concentration as compared to 0.75 gL^{-1} at 100 gL^{-1} initial xylose. As the concentration of xylose increased, ethanol specific productivity (ν) decreased from 0.98 to 0.70 h^{-1}. In these experiments a maximum yield of 0.50 (g ethanol g^{-1} xylose) was achieved with a productivity of 0.73 $gL^{-1}\,h^{-1}$. Ethanol inhibition studies suggested that the maximum tolerance of the culture was 50 gL^{-1} ethanol. However, the maximum ethanol that could be produced was 43.5 gL^{-1}. In pH controlled experiments, the maximum ethanol productivity of 0.90 $gL^{-1}\,h^{-1}$ was obtained. The value of K_m (Michaelis–Menten constant) was evaluated to be 4.38 gL^{-1}.

Keywords: ethanol; xylose; inhibition; Escherichia coli FBR5; productivity; yield.

INTRODUCTION

Current US production of fuel ethanol is 11 billion litres per year (2003; Industrial Biprocessing, 2004) and this is expected to grow to 20 billion litres per year by 2020. Increased production of ethanol is favoured by environmental, economic and national security concerns. The expected production of 20 billion litres per year can be reached by using corn as substrate. However, a further increase in ethanol production would likely necessitate using other agricultural substrates in addition to corn, such as corn fibre, corn stalks, wheat and rice straw, switch grass and other agriculture based residues. Successful use of these substrates would result in economic production of ethanol as it is anticipated that the cost of sugars derived from them would be less than the cost of glucose obtained from starch.

In previous reports, it has been demonstrated that the cost of substrate is one of the most influential factors that affect price of fuels and chemicals production from renewable resources (Marlatt and Datta, 1986; Qureshi and Manderson, 1995; Qureshi and Maddox, 1992). However, as is the case with starch, agricultural residues must be hydrolysed prior to their efficient fermentation to ethanol. It is well established that agricultural residues contain hexose and pentose sugars (Grohmann and Bothast, 1997), both of which must be converted to ethanol for the process to be economic. Genetic engineering techniques have made it possible to develop strains that efficiently utilize both hexose and pentose sugars for ethanol production, thus making ethanol fermentation of agricultural residues a viable option (Ingram *et al.*, 1987; and reviewed in Dien *et al.*, 2003).

E. coli FBR5 is a genetically and phenotypically stable strain capable of fermenting hexoses and pentoses. It was developed to produce ethanol in high yields from corn fibre hydrolysate and other agricultural residues (Dien *et al.*, 2000). Because of this, *E. coli* FBR5 is considered

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to be a superior strain with a potential to be used at industrial scale (Dien *et al.*, 2000). The objective of these investigations was to characterize ethanol production from xylose using *E. coli* FBR5. Fermentation parameters, such as tolerance to increased level of xylose, and ethanol were evaluated. Because hydrolysates from agricultural residues are frequently high in salts, effect of inhibitory sodium chloride was also evaluated. Kinetic parameters such as K_m (Michaelis–Menten constant, gL^{-1}), μ (specific growth rate constant, h^{-1}), μ_{max} (maximum specific growth rate constant, h^{-1}), and ν (specific rate of ethanol production, h^{-1}) were also evaluated. The data obtained from these evaluations will help in determining the suitability of *E. coli* FBR5 for commercial application or further commercial development. For the reasons listed in this paragraph, these characterization studies are considered to be novel.

MATERIALS AND METHODS

Microorganism and Cell Growth

Escherichia coli FBR5 was developed in our laboratory as reported previously (Dien *et al.*, 2000). The strain was maintained on modified Luria–Bertani (LB) solid medium containing 4 gL^{-1}xylose (Sigma Chemicals, St. Louis, MO, USA), 5 gL^{-1}yeast extract (Sigma Chemicals), 10 gL^{-1}tryptone (Becton Dickinson & Co., Sparks, MD, USA), 5 gL^{-1}NaCl (Sigma Chemicals), and 15 gL^{-1} agar (Sigma Chemicals) supplemented with 20 $\mu g\ mL^{-1}$ tetracycline (Sigma Chemicals) by weekly transfers on agar plates. A solution of yeast extract, tryptone, agar, and NaCl was autoclaved at 121°C for 15 min followed by cooling to approximately 45°C. At that stage, xylose and tetracycline were added from filter sterilized stock solutions (xylose 400 gL^{-1}, tetracycline 20 $mg\ mL^{-1}$) stored at 4°C to the above concentrations. The culture was maintained on LB solid medium at 4°C and was transferred to a new plate each week. In order to grow culture in liquid medium, a colony was transferred from an agar plate to 10 mL sterile liquid medium of following composition contained in a 15 mL screw capped test tube. The liquid medium for inoculum preparation contained 10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} NaCl, 1 gL^{-1} sodium acetate supplemented with 20 gL^{-1} xylose and 20 $\mu g\ mL^{-1}$ tetracycline. Filter sterilized xylose and tetracycline were added after the medium had cooled to room temperature. The test tube culture was grown at 35°C for 24 h. After growth, 5 mL of culture was used to inoculate 100 mL sterile medium.

Fermentations

Fermentation studies were performed either in 125 mL screw capped bottles containing 100 mL medium or in a 2L bioreactor. In the fermentation medium, xylose and tetracycline concentrations were 100 gL^{-1} and 20 $\mu g\ mL^{-1}$, respectively. The bottles were inoculated with 5 mL of actively growing 16–18 h-old culture followed by incubating at 35°C. Samples were taken routinely until fermentation stopped. In order to take a homogeneous sample, bottles were shaken gently prior to sampling. To study the effect of salt concentration, different amounts (0–40 gL^{-1}) of NaCl were added to the medium prior to autoclaving. Phosphate stock buffer (1.6 M, pH 6.9, 8× stock) was prepared by dissolving 8.61 g sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) and 13.86 g of sodium phosphate dibasic (Na_2HPO_4) in distilled water. The volume of the buffer solution was brought up to 100 mL. It was ascertained that the pH of the buffer solution was 7.0. The buffer solution was sterilized by autoclaving at 121°C for 15 min. In order to regulate pH in bottles, various amounts of buffer solution (3–12 mL) were added to the cooled medium prior to inoculation. Attempts were made to keep xylose concentration and liquid volume equal in all the bottles (xylose 100 gL^{-1}, vol 100 mL) except where effect of various xylose concentrations was studied (xylose 50–250 gL^{-1}; vol 100 mL). Inhibitory effects of added ethanol on cell growth (termed as G) and ethanol production (termed as P) were studied. In cell growth inhibition studies (G), the cultures were challenged with ethanol (0–50 gL^{-1}; concentration in fermentation broth) soon after inoculation, and samples were taken until both growth and fermentation had stopped. For ethanol production inhibition studies (P), cultures were challenged after 24 h of inoculation when growth had occurred. The ethanol that was used to challenge the cultures was 100% (v/v).

Bioreactor

Experiments with pH control were performed in a 2 L New Brunswick Bioreactor (BIOFLO 3000; New Brunswick Scientific Co., New Brunswick, NJ, USA). Fermentation pH was controlled at 6.5 using 4M KOH solution. The potassium hydroxide solution was not sterilized. In order to prepare reactor for fermentation, a nutrient solution was prepared containing 10 g tryptone, 5 g yeast extract and 5 g NaCl in 100 mL distilled water and autoclaved at 121°C for 15 min. One hundred g of xylose was dissolved in 900 mL of distilled water in the bioreactor. The bioreactor was autoclaved at 121°C for 15 min followed by cooling it by sparging oxygen free nitrogen gas (~60–80 $mL\ min^{-1}$). At that stage, agitation was initiated at 150 rpm. Upon cooling, the nutrient solution (100 mL) was added to the reactor and pH was brought up to 6.5 prior to inoculation. The reactor was inoculated with 50 mL of actively growing culture of *E. coli* FBR5 and both agitation and nitrogen sparging were continued for 24 h. At that stage (24 h), nitrogen sparging was stopped as the culture was producing its own gas. Agitation was continued at the same rate. One mL samples were taken intermittently followed by centrifugation (Eppendorf centrifuge 5417; Hamburg, Germany) at 15 000 g for 2 min. Clear liquid was stored at −20°C until ready for xylose and ethanol estimation.

Analyses

Cell concentration was measured using a predetermined correlation between dry weight cell concentration (105°C) versus optical density. Optical density was measured at 540 nm prior to centrifuging the cells. It was ascertained that medium components did not interfere with optical density measurements. Ethanol concentration was measured by GC (6890N; Agilent Technologies, Wilmington, DE, USA) using a packed glass column [Phase—10% Carbowax

20 M, 0.01% H_3PO_4; Support—80/100 Chromosorb WAW; size 182.88 cm × 2 mm (6 ft × 2 mm); obtained from Sigma Chemicals, St. Louis, MO, USA]. Prior to injection to the GC, samples were thawed and homogenized by mixing on a mixer (Maxi MixII; Barnstead, Dubuque, IA, USA) and diluted 10-fold with distilled water. One mL of sample was mixed with 0.1 mL of internal standard (n-propanol) before injection. The results were automatically computed using a ChemStation software package (supplied by Agilent Technologies, Wilmington, DE, USA). The samples were injected using an automatic injector. Xylose and fermentation acid (acetic, lactic, succinic and formic) concentrations were determined using Surveyor HPLC equipped with an automatic sampler/injector (Thermo Electron Corporation, West Palm Beach, FL, USA). The HPLC unit included a Surveyor LC pump and a RI-150 detector. The column (Aminex Resin-based) was obtained from BioRad (Hercules, CA, USA). The column temperature was controlled at 65°C using an external column heater (CH-30) equipped with a controller (TC-150; FIAtron, Oconomowoc, Wisconsin, USA). HPLC solvent was prepared by mixing 300 μL of concentrated sulfuric acid in 1 L deionized water. The solvent flow rate was maintained at 0.6 mL min^{-1}. The results were computed using an automatic integrator.

Ethanol productivity was calculated as concentration in gL^{-1} divided by the total fermentation time in h and is expressed in $gL^{-1}\,h^{-1}$. Ethanol yield ($Y_{p/s}$) was calculated as the total ethanol produced (g) divided by total xylose utilized (g). Specific growth rate constant (μ) values were calculated from runs where pH was regulated with buffer (9–12 mL). In order to calculate μ, a growth curve was plotted between dry weight cell concentration (called X) and time (T or t). On this curve tangents were drawn at various points (starting from end of lag phase to the end of log phase) followed by calculating $\Delta X/\Delta t$. The values of $\Delta X/\Delta t$ were divided by X, where X was the cell concentration at the tangent point, to obtain μ. The μ values that have been reported here are calculated from the log phase of the curve. The values of maximum specific growth rate (were calculated similarly, except that they were obtained from the end of lag phase of the growth curve. Due to low X values in the denominator [$\mu = (1/X)\cdot(dX/dt)$] (at the end of lag phase) high μ_{max} values were obtained as compared to μ values. Specific rate of ethanol production (ν) was calculated from the ethanol production (E) versus time (t) curve by plotting tangents at various points and calculating $\Delta E/\Delta t$. To obtain ν, $\Delta E/\Delta t$ was divided by the corresponding cell concentration obtained at the point where tangent was drawn. The values presented here are average values of two observations obtained during the log phase of the production curve.

Michaelis–Menten kinetic constant (K_m) was calculated using ethanol production and xylose utilization curves and the equation $\nu = \nu_{max}\cdot S/(K_m + S)$. This equation can also be presented in the following form:

$$1/\nu = (K_m/\nu_{max})\cdot(1/S) + 1/\nu_{max} \quad (1)$$

where ν_{max} and S are maximum specific rate of ethanol production and substrate concentration, respectively.

An experiment was run where cell growth and xylose utilization were monitored to calculate values of various parameters in equation (1). From this run, three graphs were plotted: ethanol production versus time, xylose utilization versus time, and cell concentration versus time. Along the ethanol production curve various tangents were plotted to calculate $\Delta E/\Delta t$ values and from them, ν values were calculated by dividing by the corresponding X values. At various tangent points corresponding values of S were read. Using ν and S values, Lineweaver–Burk plot [equation (1)] was plotted and then K_m value was calculated. K_m is the substrate concentration at which specific rate of ethanol production is one-half of maximum value (Aiba *et al.*, 1965).

RESULTS AND DISCUSSION

Initially, a control experiment was carried out without pH control. The fermentation resulted in the production of 27.5 gL^{-1} ethanol in 97 h of fermentation [Figure 1(a)] (Fermentation stopped at 97 h). During the fermentation, pH decreased from 6.15 to 5.30 [Figure 1(b)]. At zero time, xylose concentration was 96.2 gL^{-1} which decreased to 5.4 gL^{-1}. During the fermentation, 90.8 gL^{-1} xylose was utilized thus resulting in a xylose utilization rate of 0.94 $gL^{-1}\,h^{-1}$ and an ethanol yield of 0.30. This yield is low as compared to theoretical ethanol yield of 0.51. The acids produced during the fermentation were acetic, succinic, lactic, and formic. The total concentration of acids was 3.48 gL^{-1} (Table 1; see 0 mL buffer). In this

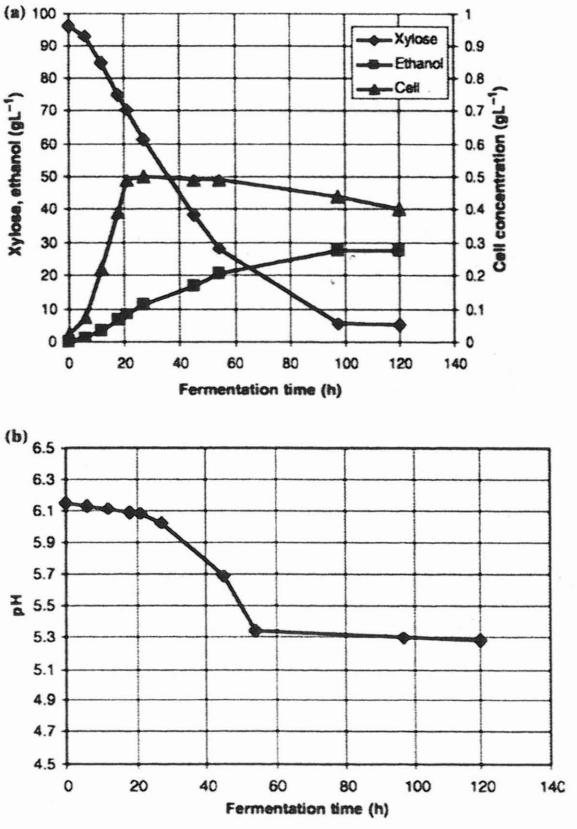

Figure 1. Production of ethanol from xylose in pH unregulated batch reactor using *E. coli* FBR5. (a) Xylose, ethanol and cell concentration; (b) final pH.

Table 1. Effect of phosphate buffer on byproducts formation during ethanol production from xylose using *E. coli* FBR-5.

	Volume of buffer added to the reactor (mL)				
By-products	0	3	6	9	12
Acetate (gL^{-1})	0.62	0.81	1.02	1.17	1.52
Succinate (gL^{-1})	1.05	1.87	1.93	2.11	2.70
Lactate (gL^{-1})	1.28	1.57	1.71	1.92	1.90
Formate (gL^{-1})	0.53	0.24	0.22	0.30	0.32
Total acids (gL^{-1})	3.48	4.49	4.88	5.50	6.44

Acetate, succinate, lactate, formate and total acids in pH controlled fermentation were 1.29, 0.11, 2.21, 0.50 and 4.11 gL^{-1}, respectively.

fermentation, an ethanol productivity of 0.28 $gL^{-1}\,h^{-1}$ was achieved, showing that this was a sluggish fermentation. A possible reason for this fermentation being sluggish was acidic pH during the fermentation. Cell growth was poor and a maximum cell concentration of 0.5 gL^{-1} was achieved. Cell growth stopped at 20 h when pH was 6.05. It is likely that a combination of reduced pH, and accumulation of byproducts and ethanol inhibited cell growth thus preventing the culture from growing further. The possible reason for low ethanol yield may have been production of acids (by-products) in the pH uncontrolled fermentation and other unknown factors. It is likely that in the absence of pH control, the culture may have been under stress thus requiring a significantly higher amount of carbon source for maintenance (Pirt, 1975; Qureshi *et al.*, 1988). Since pH was not controlled in this fermentation (which affected cell growth), μ, μ_{max}, and ν were not calculated from this run.

Effect of Buffer

Following this, an experiment was conducted in which phosphate buffer was used to regulate pH of the fermentation broth [Figure 2(a)]. The amounts of buffer that were added to the fermentation medium were 0, 3, 6, 9 and 12 mL. Addition of buffer to the reaction mixture resulted in the final pH of 5.28, 5.61, 5.80, 6.08 and 6.28, respectively [Figure 2(b)]. As a result of increase in pH values, increased amounts of ethanol were produced. At a 0 mL buffer, 27.6 gL^{-1} ethanol was produced, while at 9 and 12 mL it was 38.6 and 39.0 gL^{-1}, respectively. The amounts of used xylose at 9 and 12 mL buffer were 89.8 and 87.9 gL^{-1}, respectively [Figure 2(a)]. At these levels of buffer, fermentation time was 60 h. A maximum cell concentration of 0.89 gL^{-1} was achieved at a buffer level of 6 mL. Fermentations at 9 and 12 mL buffer levels resulted in ethanol productivities of 0.64 and 0.65 $gL^{-1}\,h^{-1}$, respectively. At 9 and 12 mL buffer levels ethanol yields of 0.43 and 0.44, respectively, were achieved. The concentrations of various acids that were produced in the fermentations increased with the increased levels of buffer (Table 1). Production of such high levels of acids, in particular at 9 and 12 mL buffer levels, may have contributed to low ethanol yield. At 12 mL buffer, the total product yield (ethanol + acids) was 0.52 which is higher than theoretical yield of ethanol (0.51). Possibly presence of carbon source in 5 gL^{-1} of yeast extract and 10 gL^{-1} of tryptone that were present in the medium, may have contributed to the overall high yield. Although at 9 and 12 mL

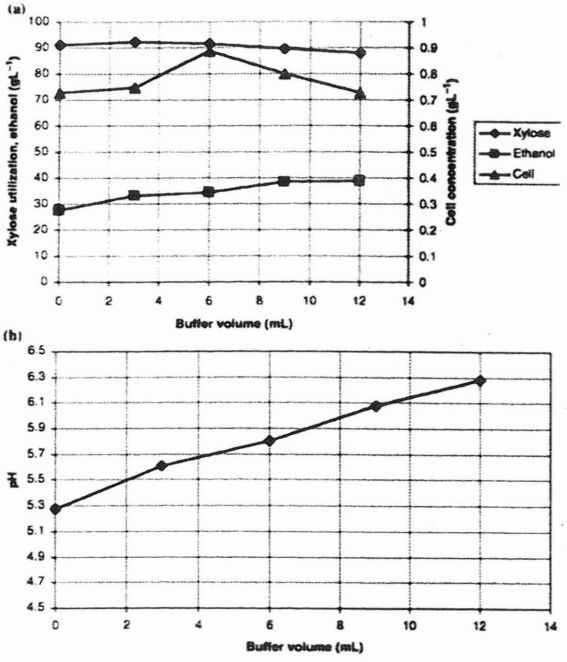

Figure 2. Production of ethanol from xylose in buffer pH regulated batch fermentations using *E. coli* FBR5. (a) Xylose utilization, ethanol production and cell concentration; (b) final pH.

buffer levels, high levels of acids were produced, still these buffer levels were considered to be optimum for ethanol production as they resulted in accumulation of higher amounts of ethanol and higher yields. Hence, further studies (including calculation of μ, μ_{max}, and ν) were performed using 9–12 mL buffer per 100 mL medium.

Effect of Osmotic Stress

It has been reported that salts are inhibitory to the cell growth and fermentation in *E. coli* (Beall *et al.*, 1991). Since our medium (Luria–Bertani broth; Dien *et al.*, 2000) contained 5 gL^{-1} sodium chloride, the effect of salt (NaCl) on growth and fermentation of *E. coli* was investigated. Additionally, salt inhibition experiments were considered relevant as acid hydrolysed agricultural biomass solutions contain salt/s. Figure 3(a) shows that a salt concentration up to 10 gL^{-1} was not inhibitory to *E. coli* cell growth and ethanol production. At that level of NaCl, the culture produced 40 gL^{-1} ethanol in 60 h of fermentation. An increase in salt concentration to 20 gL^{-1} decreased ethanol production from 40.6 gL^{-1} to 34.9 gL^{-1}. Further increases in salt concentration to 30 and 40 gL^{-1} resulted in decreased ethanol production to 26.9 and 13.9 gL^{-1}, respectively. Above 20 gL^{-1} salt concentration, cell growth decreased dramatically. At a salt concentration of 30 gL^{-1}, a cell concentration of 0.12 gL^{-1} was observed. At a salt concentration of 40 gL^{-1}, the culture used 47.9 gL^{-1} xylose. In addition to salt concentration that was used in the fermentation medium, salts present in the buffer may also have contributed to some inhibitory affects. Beall *et al.* (1991) reported accumulation of 32.0 gL^{-1} ethanol

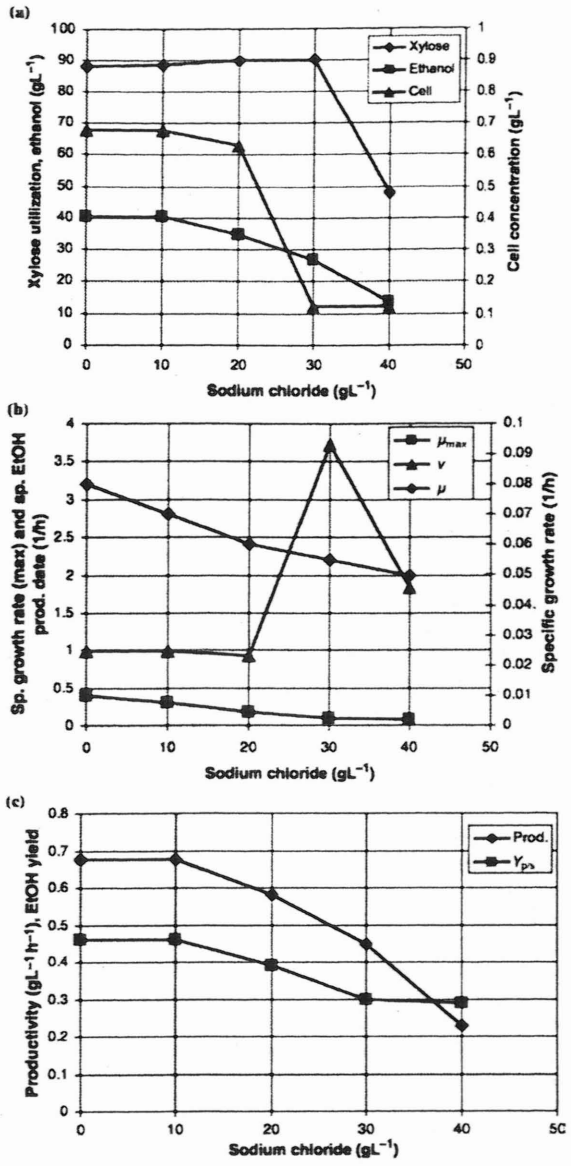

Figure 3. Effect of salt concentration on ethanol production from xylose in batch fermentations using *E. coli* FBR5. (a) Xylose utilization, ethanol production and cell concentration; (b) μ, μ_{max} and ν; (c) productivity and yield ($Y_{p/s}$).

(as compared to 56.0 gL^{-1} maximum in their experiment) when 23.4 gL^{-1} NaCl was present in the medium.

Comparison of kinetic parameters is valuable for evaluating process efficiency. Hence, kinetic parameters such as μ, μ_{max}, and ν were evaluated at various salt concentrations. The values of these parameters have been plotted in Figure 3(b). As salt concentration increased from 0 to 40 gL^{-1}, value of μ decreased from 0.08 to 0.05 h^{-1}, suggesting that rate of growth was inhibited by the presence of salt. Similarly, the value of maximum specific growth rate decreased from 0.40 h^{-1} at 0 gL^{-1} salt concentration to 0.08 h^{-1} at 40 gL^{-1} salt concentration. The value of ν decreased from 0.99 to 0.92 h^{-1}, as salt concentration increased from 0 to 20 gL^{-1}. Further increase in salt concentration to 30 gL^{-1}, resulted in a dramatic increase in specific rate of ethanol production to 3.73 h^{-1} which is over three times the specific rate of ethanol production at 0 gL^{-1} salt concentration. An increase in salt concentration to 40 gL^{-1} decreased specific rate of ethanol production to 1.83 h^{-1}. It should be noted that even though cell concentrations were low at 30 and 40 gL^{-1} salt concentrations, the rate of production of ethanol per g cell mass increased (ν) thus using less carbon for cell growth. Both ethanol productivity and yield decreased above a salt concentration of 10 gL^{-1} [Figure 3(c)]. The reason for decreased productivity was reduced cell growth due to salt inhibition and reduced yield was due to cell maintenance, which will be discussed later.

Integrated systems are prolonged fermentations and hence tend to accumulate salts thus inhibiting the culture. In order to keep inhibitory salt level below the toxic limit, a small bleed from the fermentor would be required. Keeping in view ethanol concentration (that can be achieved in fermentation broth), productivity, and yield, the total salt concentration should be kept below 10 gL^{-1} in the integrated experiments to avoid growth and product formation inhibition. It should be noted that increased salt concentration suppressed acid production (Table 2), however, it was at the expense of both reduced productivity and yield [Figure 3(c)].

It should be noted that μ_{max} values were much higher than the μ values. The μ values were calculated starting from the end of lag phase (when cell growth started) to the end of log phase (at various points during log phase). The values that were calculated from the end of lag phase were the highest and hence they were called μ_{max}. For an experiment with 0 gL^{-1} NaCl, the $\Delta X/\Delta t$ values at the end of lag phase, and during the log phase were 0.020 and 0.025 $gL^{-1}\,h^{-1}$, respectively. When these values were divided by the corresponding X values, 0.05 and 0.315 gL^{-1}, the resulting μ_{max} and μ values were 0.40 and 0.079 (or 0.08) h^{-1}, respectively. Similar differences in μ_{max} and μ values were observed when these parameters were calculated at various initial xylose levels in the medium (see below).

Xylose Tolerance

Further, experiments were conducted to investigate xylose tolerance level of the culture. To achieve this, initial xylose concentrations ranging from 50 to 250 gL^{-1} were used in the fermentation vessels (Figure 4). Fermentation with an initial xylose concentration of 50 gL^{-1} was

Table 2. Effect of sodium chloride on byproducts formation during ethanol fermentation using *E. coli* FBR-5.

	Sodium chloride concentration (gL^{-1})				
By-products	0	10	20	30	40
Acetate (gL^{-1})	0.90	0.73	0.91	1.01	1.02
Succinate (gL^{-1})	1.36	1.36	0.66	0.42	0.30
Lactate (gL^{-1})	2.52	2.00	1.74	0.70	0.52
Formate (gL^{-1})	0.37	0.32	0.00	0.53	0.16
Total acids (gL^{-1})	5.15	4.41	3.31	2.66	2.00

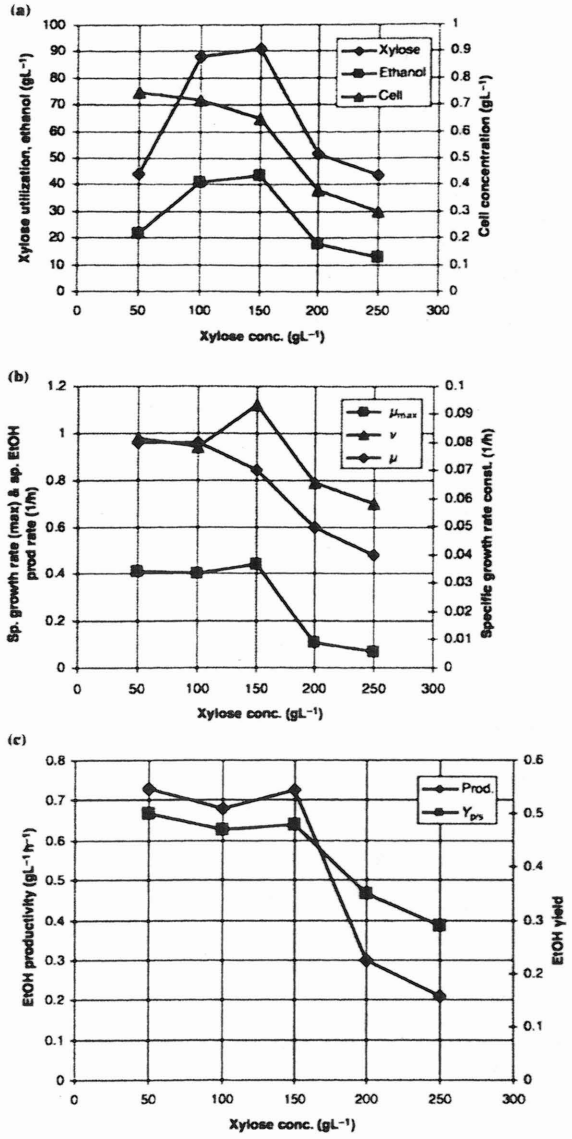

Figure 4. Effect of xylose concentration on ethanol production in batch fermentations using *E. coli* FBR5. (a) Xylose utilization, ethanol production and cell concentration; (b) μ, μ_{max} and ν; (c) productivity and yield.

complete in 30 h, while other fermentations were run for 60 h. The ethanol concentrations that were achieved in these fermentations ranged from 12.7 to 43.5 gL^{-1} [Figure 4(a)]. Xylose utilization at various initial xylose levels of 50, 100, 150, 200 and 250 gL^{-1} were 44.0, 88.0, 91.0, 51.7 and 43.7 gL^{-1}, respectively. The culture experienced a strong growth inhibition due to increased level of xylose in the fermentation broth. Above 150 gL^{-1} of initial xylose, cell growth was strongly inhibited. At 250 gL^{-1} xylose, cell concentration decreased to 0.30 gL^{-1}. The reduced cell growth may have been due to increased osmotic pressure at high xylose concentrations. As with increased xylose levels, significantly reduced cell growth was observed when 20–40 gL^{-1} NaCl was used (previous section) in the medium. It is likely that NaCl also exerted an osmotic pressure on the cell. However, there may have been other mechanisms for growth inhibition by NaCl.

Use of concentrated substrate resulted in a significant level of unused xylose. However, such fermentations can be integrated with suitable product recovery technique to utilize xylose completely as demonstrated for solvent producing Clostridia (Ezeji *et al.*, 2003; Maddox *et al.*, 1995; Maddox, 1989). Application of integrated fermentations, where concentrated sugar solutions are used, results in better economic processes (Qureshi and Blaschek, 2001; Qureshi and Maddox, 1992). In such processes, waste disposal loads are reduced along with reduction in energy requirement for processing the feed stream and concentration and purification of the product.

As with the sodium chloride experiment, kinetic parameters such as μ, μ_{max}, and ν were evaluated at various initial xylose concentrations. At an initial xylose concentration of 50 gL^{-1}, a value of μ of 0.08 h^{-1} was obtained [Figure 4(b)]. Increasing xylose concentration above 100 gL^{-1} resulted in a constant decrease of μ. However, μ_{max} remained nearly constant, with a value of 0.40 to 0.44, up to an initial xylose concentration of 150 gL^{-1}. Further increases in initial xylose concentration resulted in a decrease of μ_{max} value. As was the case with sodium chloride (30 gL^{-1}), ν increased from 0.98 to 1.12 h^{-1} before declining, indicating that the cell produced more ethanol per g cell per h. This suggested that cells that survived inhibition (in case of both NaCl and Xylose) produced ethanol efficiently. The actual reason for this phenomena is not clear, however it is suggested that an analysis be performed of the enzyme activities that involve ethanol production under these conditions. This phenomenon (increased ν) may result in ethanol production more efficiently when employed at industrial level. It is likely that under increased osmotic pressure caused by NaCl or xylose, cell required more energy for survival. In order to obtain that energy the cell produced ethanol at a faster rate than under normal conditions. It should be noted that in both of these cases (NaCl and xylose) reduced ethanol yield was obtained. Studies with ethanol productivity and yield were also conducted at various initial xylose concentrations [Figure 4(c)].

Up to an initial xylose concentration of 150 gL^{-1}, ethanol productivity remained between 0.68 and 0.73 $gL^{-1}h^{-1}$, which decreased to 0.21 $gL^{-1}h^{-1}$ at an initial xylose concentration of 250 gL^{-1}. Similarly, ethanol yield was decreased from 0.50 to 0.29 as xylose concentration increased from 50 to 250 gL^{-1}, respectively. During these studies, fermentation by-products were also measured. At an initial xylose concentration of 50 gL^{-1}, 1.93 gL^{-1} total acids were produced (Table 3). The total

Table 3. Production of byproducts during ethanol fermentation at various xylose concentrations.

	Initial xylose concentrations (gL^{-1})				
By-products	50	100	150	200	250
Acetate (gL^{-1})	0.67	1.13	0.86	0.68	0.67
Succinate (gL^{-1})	0.57	2.06	0.75	0.60	0.42
Lactate (gL^{-1})	0.50	2.01	0.69	0.46	0.32
Formate (gL^{-1})	0.19	0.19	0.00	0.00	0.00
Total acids (gL^{-1})	1.93	5.39	2.30	1.74	1.41

acids at 100 gL^{-1} initial xylose were at 5.39 gL^{-1} which decreased to 1.41 gL^{-1} as the concentration of xylose increased to 250 gL^{-1} (Table 3).

The full potential of an integrated process could be achieved if ethanol is produced and recovered simultaneously. In such cases, concentrated sugar solutions can be fermented to produce ethanol. The authors are aware that agricultural biomass hydrolysates contain sugar concentrations ranging from 60–100 gL^{-1} which is good only for non-integrated batch fermentations. However, sugar concentration in the hydrolysate can be increased by two techniques: firstly, by removing water prior to fermentation; and the second, by supplementing with glucose obtained from a cornstarch processing plant. In the first case, the process would be uneconomic as evaporation of water would require a significantly higher amount of energy. The second process would be attractive as reactor productivity would be improved and cells and water would be recycled as demonstrated by Ezeji *et al.* (2003, 2004). *E. coli* FBR5 is capable of fermenting glucose and xylose mixtures simultaneously as demonstrated by Dien *et al.* (2000).

End Product Inhibition

Next, growth inhibition studies (G) were performed and the culture was challenged with different ethanol concentrations [Figure 5(a)]. In this experiment, the objective was to investigate the effect of added ethanol (added at zero time) on cell growth, xylose utilization [xylose (G)] and ethanol production [ethanol (G)]. At 0 gL^{-1} of added ethanol, the culture produced 0.74 gL^{-1} of cell mass, which decreased as the ethanol concentration was increased in the fermentation medium [Figure 5(b)]. At an added ethanol concentration of 50 gL^{-1}, the culture did not grow. Specific growth rate decreased linearly as the concentration of added ethanol was increased from 0 to 50 gL^{-1} [Figure 5(b)]. At 0 gL^{-1} ethanol concentration in the medium, 88.0 gL^{-1} xylose [xylose (G)] was used by the culture thus producing 41.3 gL^{-1} ethanol [EtOH (G)]. At 10 gL^{-1} ethanol in the medium, 66.7 gL^{-1} xylose was used and 31.2 gL^{-1} ethanol was produced. At an added ethanol concentration of 50 gL^{-1}, neither xylose was used nor was ethanol produced, suggesting that at 50 gL^{-1} added ethanol there was complete inhibition. Ethanol productivity [Prod (G)] and ethanol yield [Yield (G)] obtained in this experiment are presented in Figure 5(c). Ethanol productivity decreased nearly linearly as the concentration of added ethanol increased. Ethanol yield also decreased with the increase in added ethanol. At an added ethanol concentration of 40 gL^{-1}, ethanol yield of 0.35 was obtained while it was 0.47 with no added ethanol concentration. The concentration of acids that were in this fermentation decreased with the added ethanol (Table 4). At no added ethanol, total acids were at 5.31 gL^{-1}, while at 40 gL^{-1} added ethanol, acids were at 0.72 gL^{-1}.

It is possible that initial ethanol added, affected the fermentation by retarding initial growth and not exclusively through end-product inhibition. To investigate this possibility, another experiment was carried out in which the culture was challenged with ethanol at various concentrations after 24 h of growth. In this case, ethanol production [Prod (P)], xylose utilization [xylose (P)], ethanol productivity [Prod (P)], and yield [Yield (P)] were measured and compared with the growth inhibition experiment (G). Results from the two experiments were similar [Figure 5(a)–(c)]. Since data on cell growth and μ were similar, they have not been plotted.

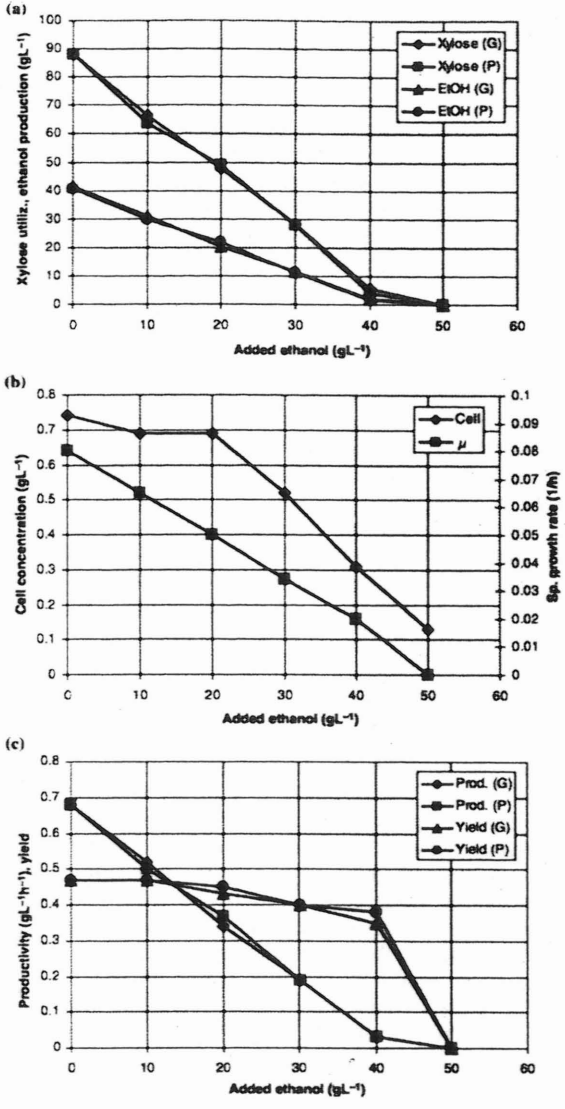

Figure 5. Effect of added ethanol inhibition on cell growth and ethanol production by *E. coli* FBR5. (a) Xylose utilization and ethanol production; (b) cell concentration and μ; (c) productivity and yield.

Table 4. Effect of added ethanol on byproducts formation during ethanol production from xylose using *E. coli* FBR-5.

	Added ethanol concentration (gL^{-1})				
By-products	0	10	20	30	40
Acetate (gL^{-1})	1.08	0.86	0.44	0.28	0.13
Succinate (gL^{-1})	2.03	2.61	1.25	0.92	0.46
Lactate (gL^{-1})	1.95	1.75	0.38	0.36	0.13
Formate (gL^{-1})	0.25	0.00	0.00	0.16	0.00
Total acids (gL^{-1})	5.31	5.22	2.07	1.72	0.72

Automatic pH Control Fermentation

Further, in order to compare experiments where pH was regulated by buffer, and by automatic pH control by base (KOH) addition, an experiment was conducted with controlled pH at 6.5. In this experiment, initial xylose was 90.1 gL^{-1} (Figure 6). At the end of fermentation 42.5 gL^{-1} ethanol was produced, resulting in a productivity and yield of 0.9 gL^{-1} h^{-1} and 0.48, respectively (fermentation stopped at 47 h). These results are comparable to the data where pH was regulated using buffer. In this experiment, a cell concentration of 1.83 gL^{-1} was achieved. The concentration of total acids was 4.11 gL^{-1} (Table 1; see footnote).

It has been observed that under extreme conditions (high salt, xylose, and ethanol concentrations) ethanol yield was found to be low in spite of the fact that these conditions resulted in reduced production of by-products. Additionally, biomass concentration was also low. Under these conditions (reduced by-product formation and reduced cell growth) ethanol yield should have been higher than observed as less carbon was used for by-product formation and cell growth. In fermentation, the culture utilizes the carbon source for cell growth, ethanol production, acids production, cell maintenance, and for the production of unknown products. Since the culture used less xylose for growth and by-product formation, the only possible explanation remains that a significant amount of the carbon source was used for the production of unknown chemicals and for cell maintenance, and hence a lower yield. An example of production of unknown chemicals is accumulation of trehalose by *E. coli* to maintain turgor pressure for cell survival under high external osmolality such as high salt or sugar concentration (Strom *et al.*, 1986). Maintenance of turgor pressure is essential for growth and division of the cell (Ingraham and Marr, 1996). It has been proposed that the stress of turgor pressure on the bacterial cell wall is instrumental in the enlargement of the cell wall (Koch, 1983). Turgor pressure is communicated from the cytoplasmic membrane to the cell wall either directly or, in bacteria with a periplasm by means of a gel composed of highly hydrated, uncross-linked strands of peptidoglycan that fill space between the cytoplasmic membrane and the wall (Hobot *et al.*, 1984). In addition to accumulation of trehalose, glutamate and K^+ are also accumulated (Ingraham and Marr, 1996) to maintain the cell activity. In a more recent study, it has been demonstrated that under anaerobic conditions the amount of proline (osmolyte) was much higher (71.9 nmol mL^{-1}) than any other osmolytes and cell growth was limited (Underwood *et al.*, 2004). It is also likely that some cell components are produced when cell is exposed to high ethanol concentration. Additionally, fermentation is often complex and there may have been other chemicals that were produced and remained undetected by GC or HPLC under the conditions these machines were operated to measure ethanol, acids, and sugar. These are the only explanations that can be given for the reduced product yield under extreme conditions. In order to improve yield (under regular conditions; not extreme conditions), acid production should be reduced.

Kinetic Parameters Evaluation

Evaluation of kinetic parameters is essential for process scale-up. Since, *E. coli* FBR-5 has potential to be used at a large scale due to culture's stability in continuous reactors, high ethanol yield, and ability to utilize glucose, xylose, and arabinose, kinetic parameter such as K_m was evaluated using xylose as substrate. The value of K_m was 4.38 gL^{-1} of xylose (Table 5). In view of the objectives of these studies, the culture has been characterized for its tolerance to sodium chloride, xylose, and ethanol. These tolerance levels would be useful in planning further experiments on recovery of ethanol in an integrated process where water is recycled to the bioreactor (Qureshi and Blaschek, 2001; Ezeji *et al.*, 2003). Since *E. coli* can tolerate a xylose concentration up to 250 gL^{-1}, concentrated xylose or mixed sugar batch experiments with product recovery would be performed. Use of concentrated sugar solution would be beneficial to the process as it would reduce process stream, reduce waste disposal loads, result in higher reactor productivity, and recycle water or spent fermentation broth (Ezeji *et al.*, 2003, 2004; Qureshi and Blaschek, 2001; Friedl *et al.*, 1991).

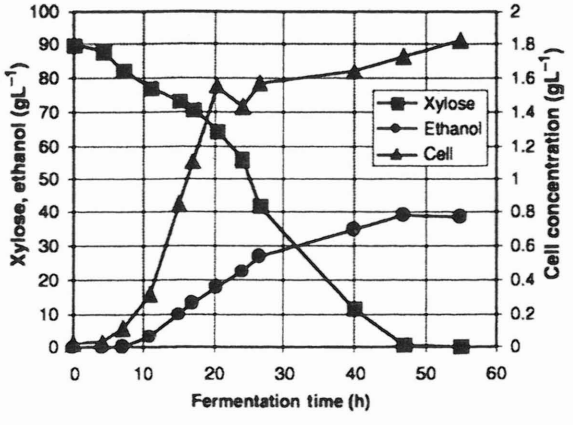

Figure 6. Production of ethanol from xylose in pH controlled batch bioreactor using *E. coli* FBR5.

Table 5. Kinetic parameters of ethanol production from xylose using *E. coli* FBR-5.

Kinetic parameters	No pH control	Buffer (9 mL)	pH control
Ethanol (gL^{-1})	27.6	38.6	42.5
Max cell conc (gL^{-1})	0.50	0.89	1.83
Xylose utilization (gL^{-1})	91.1	89.8	90.1
Productivity (gL^{-1} h^{-1})	0.28	0.64	0.90
Yield (gg^{-1})	0.30	0.43	0.47
Fermentation time (h)	97	60	47
Xylose tolerance (gL^{-1})	150–250		
Ethanol tolerance (gL^{-1})	50.0		
Optimum NaCl (gL^{-1})	10		
Max ethanol produced (gL^{-1})	43.5		
Max yield (gg^{-1})	0.47–0.50		
Max batch productivity ($gL^{-1}h^{-1}$)	0.90		
K_m (gL^{-1})	4.38		

CONCLUSIONS

In conclusion, *E. coli* FBR5 is inhibited by high concentration of sodium chloride. A salt concentration of greater or equal to 10 gL^{-1} does not result in inhibition. The maximum concentration of salt that *E. coli* can tolerate is approximately 40 gL^{-1}. At that concentration the value of μ_{max} is reduced significantly due to inhibition. Studies on the effect of salt concentration on cell growth and ethanol production were performed for two reasons: first, acid hydrolysates contain salts and hence these inhibition studies become essential; and second, to study simultaneous ethanol production and recovery from the broth and recycle water or spent fermentation broth to the fermentor. Such integrated systems have proved to be beneficial to the economics of chemical and fuel production by fermentation (Qureshi and Blaschek, 2001; Friedl *et al.*, 1991).

The culture can tolerate a maximum xylose concentration of 250 gL^{-1}, however, at that concentration a reduced cell growth was observed. A maximum cell concentration of 0.30 gL^{-1} was obtained at this sugar concentration as compared to 0.75 gL^{-1} at 100 gL^{-1} initial xylose. As the concentration of xylose increased, the value of ν decreased from 0.98 to 0.70 h^{-1}. In these experiments a maximum yield of 0.50 was achieved with a productivity of 0.73 $gL^{-1}\,h^{-1}$ (Table 5). Ethanol inhibition studies suggested that the maximum tolerance of the culture was 50 gL^{-1}. However, the maximum ethanol that could be produced was 43.5 gL^{-1}. In pH controlled experiments, the maximum ethanol productivity of 0.90 $gL^{-1}\,h^{-1}$ was obtained. The value of K_m was estimated to be 4.38 gL^{-1} (Table 5). Ethanol yield of 0.41–0.51 and ethanol accumulation of 16.7–26.7 gL^{-1} have been reported using other *E. coli* strains (Lawford and Rousseau, 1995, 1996). Using *Zymomonas mobilis* AX101 ethanol yield of 0.31–0.51 and ethanol accumulation of 10.3–46.3 gL^{-1} were obtained (Lawford and Rousseau, 2002).

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